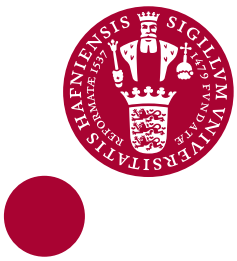

Somatic embryogenesis and in-vitro regeneration of rice (Oryza sativa L.) cultivars under one-step and multiple-step salinity stresses

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SOMATIC EMBRYOGENESIS AND *IN-VITRO* REGENERATION OF RICE (*ORYZA SATIVA* L.) CULTIVARS UNDER ONE-STEP AND MULTIPLE-STEP SALINITY STRESSES

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A B S T R A C T

The present study aimed to examine the effect of one-step and multiple-step salinity stress on the somatic embryogenesis of rice cultivars within the solid and liquid (cell suspension) culture media conditions. Five rice cultivars, including Puteh Perak, Mahsuri, Basmati-370, Nona Bokra and Khari Gunja were used in this study. The callus cultures were induced from the rice seed embryo using solid MS media containing 10 µM 2,4-D and 2 µM Kinetin. The results indicated that in the one-step NaCl treatment, the growth of the control calli and cell suspensions of the cultivars was decreased, and significant morphological changes were observed. In contrast, the multiple-step NaCl treatment of the calli and cell suspensions led to higher growth of the cultures in the presence of NaCl compared to the controls. The solid MS media, containing 3 µM IAA and 40 µM Kinetin performed as the best media for plant regeneration in both calli and cell suspensions. The regeneration capacity of the one-step treated calli and cell suspensions were decreased with the increased concentration of NaCl in the media. Higher regeneration frequencies occurred in the multiple-step treated calli of Pueth Perak and Nona Bokra compared to their controls while the other cultivars showed a lower regeneration under the same trend of salinity. The plant regeneration capacity of the multiple-step treated, as well as the control cultures, was decreased with increasing the cultures age. However, the NaCl-treated cultures maintained higher regeneration capacity under the both modes of treatment (one-step and multi-step) for up to 48 weeks compared to the control treatments.

Keywords: Rice, somatic embryogenesis, salinity stress, kinetin, auxin.

Abbreviations: MS: Murashige and Skoog culture media, LS: Linsmaier and Skoog culture media, PGR: plant growth regulator, OS: one-step, MT: multiple-step, PP: Puteh Perk, Mah: Mahsuri, B-370: Basmati- 370, NB: Nona Borkra, KG: Khari Gunja, 2,4-D: 2,4-Dichlorophenoxyacetic acid, IAA: Indole-3-acetic acid, NAA: 1-Naphthalene acetic acid, BA: benzyl adenine, MARDI: Malaysian Agricultural Research Development Institute, PARC: The Pakistan Agricultural Research Council, ANOVA: analysis of variance, SE: standard error, CRD: completely randomized design, RCBD: randomized complete block design, cv.: cultivar, M: media, S: salinity, T: time, LBDs: Lateral Organ Boundaries Domain transcription factors, CIM: callus-inducing medium, 2-MCPP: 2-(2-methyl-4-chlorophenoxy) propionic acid, MB-MBR: moving bed-membrane bioreactor, MBR: membrane bioreactor.

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INTRODUCTION

The adverse effects of salt on the plants are expressed in two pathways; on one hand, high concentration of salt in soil immediately hampers the water absorption by roots, on the other hand, accumulation of salt in different organs, poisons the plants (Munn and Tester, 2008). The two toxic ions Na^+ and Cl^- derived from NaCl can damage the plant cells through the both osmotic and ionic mechanisms (Chinnusamy *et al*., 2005). According to Zeng *et al*., (2002), rice yield can be decreased up to 60% during growth under moderate levels of salinity. However, in the recent years, researchers aimed to develop salt-tolerant rice cultivars through classic genetic and modern biotechnological approaches by using tissue cultur teqhnices (Nam *et al*., 2014). A suitable culture medium for a particular genotype could be not suitable for another genotype thus, several optimizations need to be done to find the best media for each genotype. Similarly, the efficiency of tissue culture products can alter from one laboratory to another. Accordingly, numerous protocols have been developed with a focus on preparing the best tissue culture conditions (Wanichananan *et al*., 2010 Abiri *et al*., 2017).

Plant growth regulators (PGRs), organic components, concentration and composition of the basal salt, physiological status of the explants, and the genotype of the donor plant are the most essential factors which have the potential to induce callus and regenerate rice in tissue culture experimentations (Delporte *et al*., 2001). Auxins, abscisic acid, cytokinins, ethylene and gibberellins are the five major groups of naturally occurring plant hormones. Cytokinins, auxin and auxin-cytokinin interactions are commonly considered to be the most important factors to organize the growth regulation and development of the plant organs in tissue cultures (Vasil and Thorpe, 1994). Nevertheless, lots of the genotypes, particularly indica rice varieties, as well as some of the japonica varieties, are still recalcitrant in callus induction process (Sahoo *et al*., 2011). More importantly, it is an eminent matter to understand the quality of the salinity-derived damages under different forms of the salinity induction.

To this end, the present investigation was aimed to develop a tissue culture protocol for callus induction and plant regeneration of five Indica rice cultivars under normal and salinity conditions at different solid and liquid media. The NaCl treatment mode under two forms of one-step and multiple-step salt treatment was taken into consideration as an important factor in this study.

MATERIALS AND METHODS

Plant materials: Seeds of five Indica rice (*Oryza sativa* L.) cultivars viz. Puteh Perk, Mahsuri, Basmati- 370, Nona Borkra and Khari Gunja were obtained from the Malaysian Agricultural Research Development Institute (MARDI), and the Pakistan Agricultural Research Council (PARC). Mature rice seeds were dehusked manually and rinsed with running water for one hour. The seeds were surface sterilized with 20% Sodium hypochlorite and 0.1% (v/v) Polyoxyethylene sorbitan monooleate (tween® 80; Sigma Aldrich - USA) for 30 minutes, followed by rising to five times with sterile double-distilled water.

Tissue culture media: Three different tissue culture media, including MS (Murashige and Skoog, 1962), B5 (Gamborg *et al*., 1968) and LS (Linsmaier and Skoog, 1965) media supplemented with different concentration of 2,4-Dichlorophenoxyacetic acid (2,4-D), Indole-3-acetic acid (IAA), 1-Naphthalene acetic acid (NAA), benzyl adenine (BA), and Kinetin were surveyed for callus induction, cell suspension and regeneration in this study. The pH of all media was adjusted to 5.8. In solid media, four g L^{-1} gelrite was added while, in the liquid media, no gelrite was used.

Culture maintenance condition: After transferring the required culture (seeds or callus) to the flask under sterile conditions, they were placed in the culture room either in the dark or continuous fluorescent light (1000 Lux) at 27 ± 2 ºC. Flasks containing cell suspension cultures were placed on the gyratory shaker at 110 rpm under light as mentioned above. The relative humidity of the culture room was about 70-80%.

Statistical designs and experiments: Data were analyzed using SPSS software, version 22. Two-way analysis of variance (ANOVA) was used to determine the effects of the cultivars, PGRs and media on the frequency of the embryogenic callus formation and cell suspension initiations. ANOVA was also used to assess the effect of the one-step and multiple-step salinity induction on the formation of embryogenic callus, as well as the initiation of the cell suspension and comparison of means were conducted using Duncan's Multiple Range Test ($p \leq 0.01$). The results were expressed in terms of mean ± standard error (S.E) and n=3. The differences were considered to be statistically

significant if the probability values (p-value) were less than 0.05 ($p \leq 0.05$).

Experiment 1: The first experiment of the present study was aimed to compare the impact of different ratios of 2,4-D and Kinetin on callus induction and cell suspension cultures using the solid MS and liquid B5 media, respectively. An experiment using completely randomized design (CRD) with three replicates was conducted to find the best ratio of the mentioned PGRs by using one of the rice cultivars viz. Puteh Perak. Consequently, the seeds of Puteh Perak cultivar were surface sterilized and transferred to MS solid media supplemented with 30 g L^{-1} sucrose, 0.4 g L^{-1} casein hydrolysate, four g L^{-1} gelrite with different ratios of 2,4-D and Kinetin. To initiate the cell suspension culture, 2 g of friable embryogenic fresh calli of rice cultivar Puteh Perak was dissected into small pieces and transferred to B5 liquid media supplemented with 30 g L^{-1} sucrose, 0.4 g L^{-1} casein hydrolysate and different ratio of 2,4-D and Kinetin.

Experiment 2: The experiment 2 was implemented using a factorial design on the basis of randomized complete block design (RCBD) with three replicates concentrated on testing the performance of the five rice cultivars in terms of callus initiation and cell suspension in three different culture media, including MS, B5, and LS. To achieve this, the surface sterilized seeds of the five cultivars were placed on the MS, B5 and LS solid media. Each media was supplemented with 30 g L^{-1} sucrose, 0.4 g L^{-1} casein hydrolysate, four g L^{-1} gelrite, 10 µM 2,4-D and 2 µM Kinetin according to the results of the first experiment. The efficiency of each cultivar in callus induction was calculated after 28 days of culture, according to the methods of Suengaga *et al*., (1982) and Heszky (1986). The tissue culture and cell suspension were done by following the same procedure as mentioned in the experiment 1. For the selection of suitable media for cell suspension culture initiation of five cultivars of rice, two g of fresh embryogenic calli were transferred to MS, B5 and LS media, each containing 30 g L^{-1} sucrose, 0.4 g L^{-1} casein hydrolysate and 10 µM 2,4-D (obtained from the earlier experiment). The cultures were maintained as explained in the section of culture condition. The cell suspension was regularly examined under inverted microscope for homogenous cell suspension. After 14 days of culture, the cell suspension cultures were filtered through 710 µm stainless steel sieves under aseptic condition. The callus on the sieves was discarded, and 5 mL of the suspended cells was pipetted into the fresh B5 liquid media. The fresh and dry weights of the cell suspension of each cultivar were measured after 14 days of culture.

Experiment 3: The third experiment of the present research was concentrated on studying the impact of salinity for a certain period of time on the performance of two rice cultivars (Puteh Perak and B-370) in terms of callus initiation and cell suspension frequencies. To succeed this, an experiment was carried out using a factorial design considering three factors (salinity, cultivar and time) based on RCBD with three replicates. The nine-day-old calli and six-day-old cell suspensions were directly exposed to MS and B5 media supplemented with high NaCl containing 270 mM and 70 mM NaCl, respectively. The 0.5 g fresh weight of callus (dry weight= 0.05 g) and five mL cell suspension (dry weight= 0.04 g) were cultured on their respective NaCl-containing and NaCl-free media after each passage. The duration of each passage was 18 days for callus and 12 days for cell suspension. The callus and cell suspension dry weight was recorded after each passage for up to 14 and 10 passages, respectively. It should be declared that the number of passages was started from zero.

Experiment 4: The fourth experiment was run to investigate the impact of one-step and multiple-step salinities on the performance of all the five rice cultivars in terms of callus initiation and cell suspension frequencies. To this end, another test was performed using a factorial design based on RCBD in three replicates, which was considering the impact of two factors (salinity and cultivar). For the one-step and multiple- step NaCl treatment methods, 0.2 g of fresh callus and 5 mL suspended cells of the five rice cultivars were transferred to the solid MS and liquid B5 media containing zero, 180, 360 and 540 mM for callus induction, and zero, 70, 140 and 210 mM NaCl for cell suspension (Suenaga *et al*., 1982, Croughan *et al*., 1981 Binh *et al*., 1992). The duration of each passage was 18 days for callus and 12 days for cell suspension. In one-step approach the calli and suspended cells were separately exposed to the above-mentioned doses of NaCl, and the related analyses were limited only to that particular salinity level, while in the multiple-step method, the calli and cell suspensions of the lower NaCl concentration were subsequently subjected to the higher dose.

Experiment 5: The fifth experiment of the current investigation was tended to assess the effect of

different culture media (MS, B5, LS) supplemented with 3 µM IAA+ 40 µM Kinetin on regeneration of the five rice cultivars under normal condition. The test was including of both tissue culture and cell suspension procedures. Therefore, an RCBD -based factorial design with three replicates and two factors (media with three levels and cultivar with five levels) was employed to accomplish this objective. [The mentioned combination of the PGRs (three µM IAA+ 40 µM Kinetin) was designated based on a separate preliminary study, which aimed to find the most suitable PGRs' combination]. Therefore, a CRD-based factorial design with three replicates and two factors (media and cultivar) was employed to accomplish this objective. To comply with the above-mentioned objective, 1 g of friable embryogenic calli of each five cultivars were transferred to MS, B5 and LS media supplemented with 3 µM IAA + 40 µM Kinetin. In the case of the cell suspensions clumps, the suspensions were filtered through 710 µM stainless sieves, and finally, one g of the filtered residue was transferred to MS, B5 and LS media supplemented with three µM IAA + 40 µM Kinetin. The regeneration was observed up to 45 days of culture, and the regeneration frequencies were measured according to methods represented by Wang *et al*., (1987), and Kishor and Reddey (1986).

Experiment 6: To find out the effects of one-step increasing concentration of NaCl on plant regeneration of the five rice cultivars, the 9-day established calli were treated with zero, 90, 180, 270, 360, 450 and 540 mM NaCl in MS solid media for 18 days. Simultaneously, the 6-day established cell suspensions were treated with zero, 35, 70, 105, 140 and 175 mM NaCl in B5 liquid media for 12 days. This experiment was also carried out using an RCBD-based factorial design with two factors (salinity and cultivar) in three replicates. In both experiments, one g of fresh callus and cell suspension (in the same manner as explained in the experiment 5) were transferred to 100 mL Erlenmeyer flasks each containing MS regeneration media supplemented with three µM IAA + 40 µM Kinetin. The cultures were maintained as stated in the "culture maintenance condition" section. Similar to the experiment 4, in the one-step approach, the impact of each of the above-mentioned NaCl concentrations was separately studied.

Experiment 7: The last experiment of the present study was focused on exploring the influence of multiple-step NaCl treatment on plant regeneration of the five rice cultivars. Consequently, the 9-day old established calli were treated with NaCl under a multiple-step scheme with zero to 270 mM. Concurrently, the 6-day old established cell suspensions were treated under the same scheme from zero to 70 mM NaCl. In both experiments, one g of fresh callus and cell suspension (the calli and cell suspensions were treated under the multiple-step NaCl induction system as explained in the experiment 5) were transferred to 100 mL Erlenmeyer flasks each containing MS regeneration media supplemented with three µM IAA + 40 µM Kinetin. The cultures were maintained as stated in the "culture maintenance condition" section. The plant regeneration frequencies for both one-step and multiple-step were calculated according to the method described in the experiment 5. Similar to the experiment 4, in the multiple-step regeneration, the calli and cell suspensions of the lower NaCl concentration were subsequently subjected to the higher dose, and the calli and suspended cells from the highest NaCl concentration were finally subjected to the plant regeneration process.

RESULTS

The best combination of auxin and Kinetin for callus and cell suspension initiation: The analysis of variance (ANOVA) and Duncan's multiple range test of the PGRs optimization in solid MS and liquid B5 media showed that there is a significant difference ($p \leq 0.01$) in terms of callus and cell suspension initiation responses with respect to different ratios of 2,4-D: Kinetin (Table 1).

Table 1. Analysis of variance of the PGRs optimization in solid MS (tissue culture) and liquid B5 (cell suspension) media.

S.O.V	df	Mean square	
		Solid MS Media	Liquid B5 Media
Between group	15	0.014**	3.388**
Within group	32	0.00	0.003
Total	47	-	-

S.O.V: source of variation, df: degree of freedom, ** the mean difference is significant at the 1 % level.

The best results in terms of both callus and cell suspension initiation were produced by the ratios of 10:2 and 10:0 µM of 2,4-D: Kinetin (Figure 1 and 2).

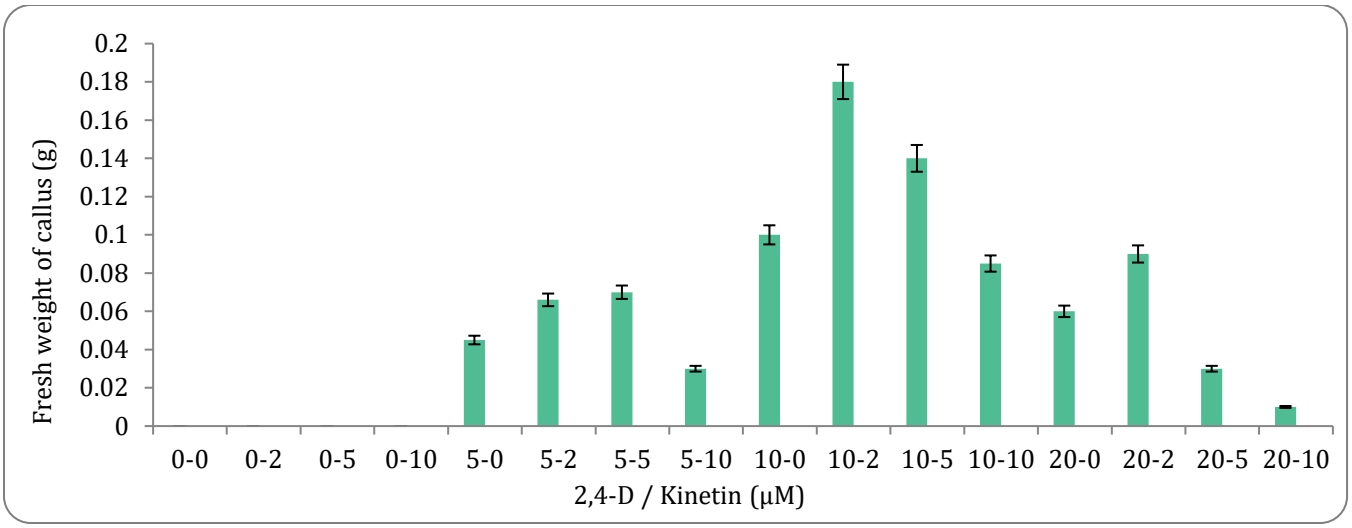

Figure 1. The effect of different concentration of 2,4-D and Kinetin on the callus induction of the Puteh Perak (PP) rice cultivar in solid MS media after 28 days of culture.

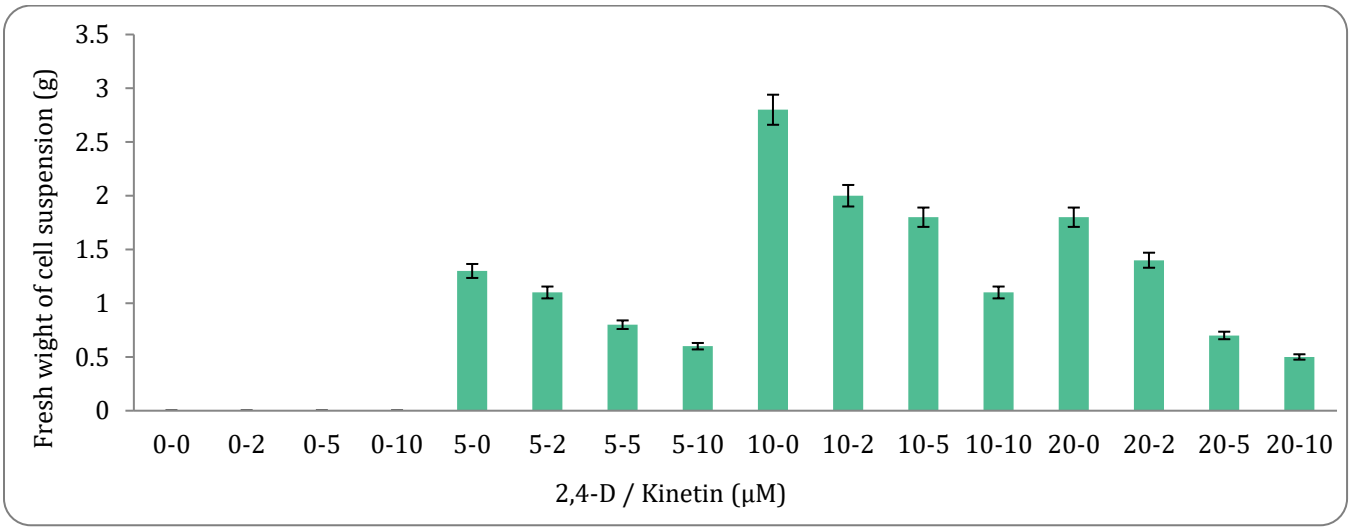

Figure 2. The effect of different concentration of 2,4-D and Kinetin on the initiation of cells suspensions of the Puteh Perak (PP) rice cultivar in B5 liquid media after 14 days of culture.

Callus and cell suspension initiation of five rice cultivars using the best combination of 2,4-D and Kinetin in MS, B5, and LS media: The ANOVA results proved that the impacts of cultivar (cv.), media (M), as well as their interaction (cv. × M) on callus and the cell suspension initiation of the five rice cultivars are all significant at 1% (Table 2).

The mean comparison of the applied treatments showed that however, MS and B5 media were the best culture media for tissue culture and cell suspension, respectively, but B-370 and KG performed as the best and worst cultivars in both culture media (Figure 3 and 4).

Table 2. Analysis of variance of callus and cell culture frequencies of five rice cultivars in different media.

S.O.V	df	Mean square	
		Tissue culture	Cell suspension
Media (M)	2	0.053**	99.090**
Cultivar (cv.)	4	0.021**	11.878**
M × cv.	8	0.002**	3.236**
Error	28	0.000	0.087
Total	42	-	-

S.O.V: source of variation, df: degree of freedom, ns: non-significant, ** the mean difference is significant at the 1% level.

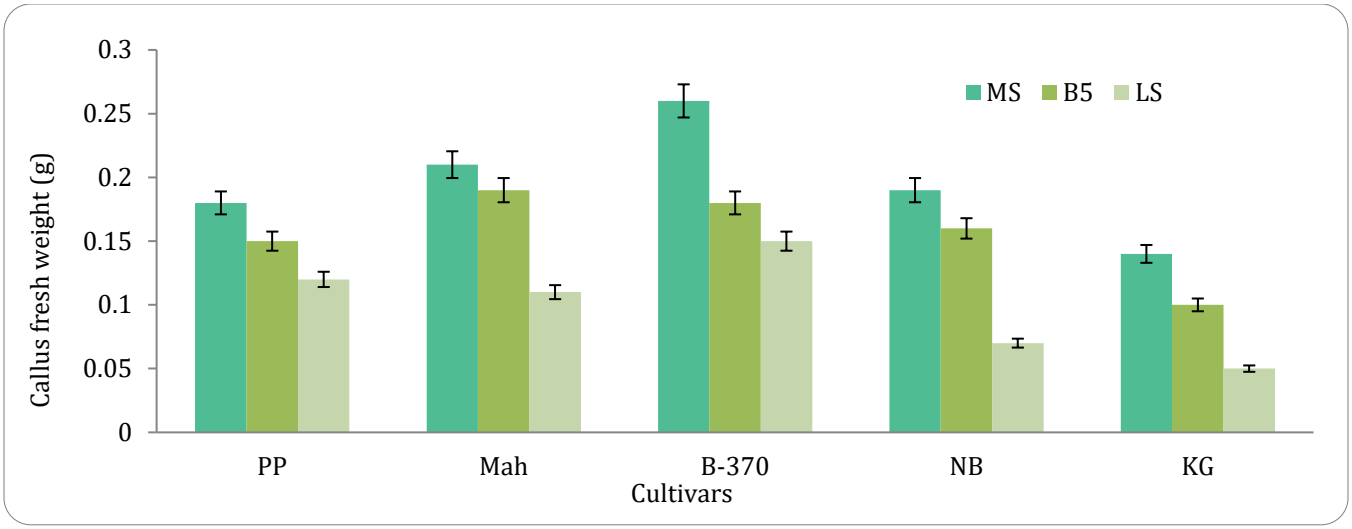

Figure 3. The effect of different media composition on the callus induction of the Puteh Perak (PP), Mahsuri (Mah), Basmati-370 (B-370), Nona Bokra (NB) and Khari Gunja (KG) rice cultivars after 28 days of culture.

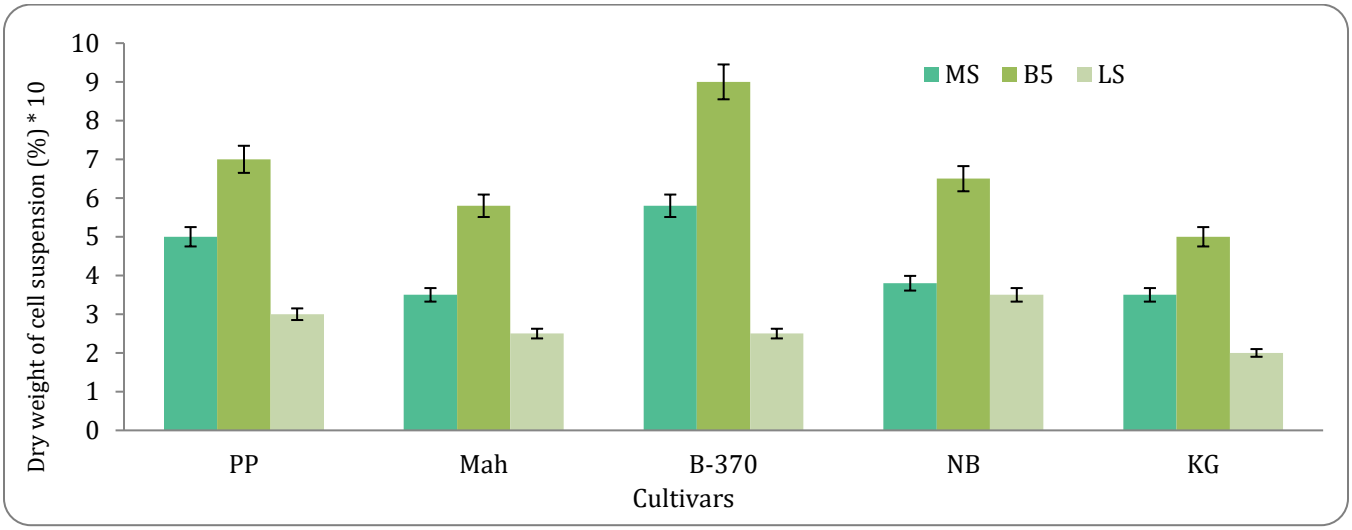

Figure 4. The effect of different media composition on the cell suspension initiation of the Puteh Peak (PP), Mahsuri (Mah), Basmati-370 (B-370), Nona Bokra (NB) and Khari Gunja (KG) rice cultivars after 14 days of culture.

The trend of callus and cell suspension initiation in different time passages under normal and salinity conditions: The ANOVA results revealed that the effect of cultivar (cv.) on the initiation of callus and cell suspension was non-significant under the one-step salinity exposure, while the effects of salinity (S) and time (T) were significant ($p \leq 0.01$) as shown in Table 3. On the other hand, the interactions of S × cv. and S × T were significant in 1% for both callus and cell suspension initiation, but the interaction of cv. × T was only significant for callus initiation and not for the cell suspension. Besides, this experiment showed that the interaction of S × cv. × T was significant on both callus and cell suspension of the used rice cultivars.

Figure 5 and 6 represent the trends of the callus and cell suspension initiation of the two rice cultivars of PP and B-370 in control and salinity conditions, respectively.

Table 3. Analysis of variance of callus and cell culture frequencies of two rice cultivars in solid MS (tissue culture) and liquid B5 (cell suspension) media in certain periods of time.

S.O.V	Tissue culture		Cell suspension	
	df	Mean square	df	Mean square
Salinity (S)	1	0.130**	1	0.026**
Cultivar (cv.)	1	0.000ns	1	0.000ns
Time (T)	13	0.057**	9	0.076**
S × cv.	1	0.021**	1	0.016**
S × T	13	0.020**	9	0.026**
cv. × T	13	0.000**	9	0.000ns
S × cv. × T	13	0.000**	9	0.000**
Error	110	0.000	78	0.000
Total	165	-	117	-

S.O.V: source of variation, df: degree of freedom, ns: non-significant, ** and * the mean difference is significant at 1 and 5% level, respectively.

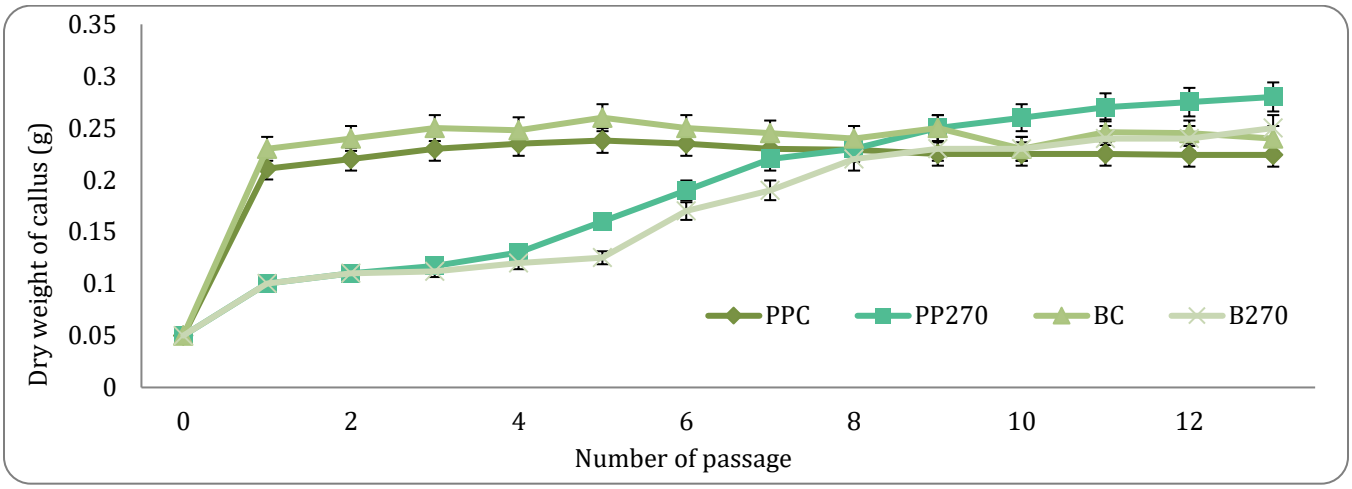

Figure 5. The long term effects of high NaCl (270 mM) on callus growth of the Pute Perak (PP) and Basmati-370 (B-370) rice cultivars. The duration of each passage was 18 days. PPC: Control treatment of Puteh Perak, PP270: Salinity treatment of Puteh Perak, BC: Control treatment of Basmati-370 and B270: Salinity treatment of Basmati-370.

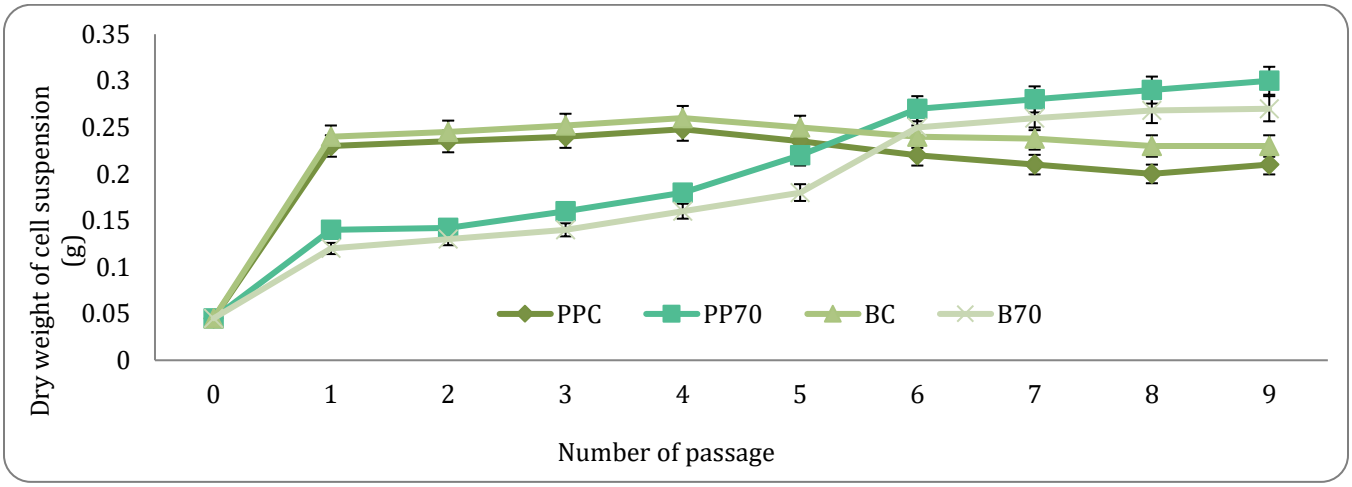

Figure 6. Long term effects of high NaCl (70 mM) on cell suspension growth of the rice cultivar Pute Perak (PP) and Basmati-370 (B-370). The duration of each passage was 12 days. PPC: Control treatment of Puteh Perak, PP70: Salinity treatment of Puteh Perak, BC: Control treatment of Basmati-370 and B70: Salinity treatment of Basmati-370.

Impact of one-step and multiple-step salinity on callus and cell suspension initiation: The ANOVA results unveiled that the effect of salinity and cultivar, as well as their interactions on the callus and cell suspension initiation of the five rice cultivars under both one-step and multiple-step salinity exposure are significant at 1% (Table 4). Consequently, the results of the Duncan test showed that the performance of the five cultivars in terms of callus and cell suspension initiation on the basis of dry weight under both one-step and multiple-step salinity exposure are significantly different ($p \leq 0.05$) as shown in Tables 5 and 6.

Table 4. Analysis of variance of callus and cell suspension frequencies of five rice cultivars in solid MS (tissue culture) and liquid B5 (cell suspension) under one-step and multiple-step salinity stress condition.

S.O.V	df	Mean square		Mean square	
		OS tissue culture	OS cell suspension	MT tissue culture	MT cell suspension
Salinity (S)	3	19276.2**	22402.15**	28131.75**	29186.8**
Cultivar (cv.)	4	189.6**	225.9**	610.275**	447.9**
S × cv.	12	22.7**	42.4**	71.875**	58.3**
Error	38	0.534	0.655	0.555	0.474
Total	57	-	-	-	-

OS: One-step salt treatment, MT: Multiple-step salt treatment, and ** the mean difference is significant at the 1 % level.

Table 5. Mean comparison of the relative growth of calli in one-step and multiple-step salinity treatment on the basis of dry weight (%).

NaCl	One-step NaCl treatment					Multiple-step NaCl treatment				
(mM)	PP	Mah	B-370	NB	KG	PP	Mah	B-370	NB	KG
0	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{c}	100^{c}	100^{c}	100^{c}	100^{c}
180	71^{b}	64^{b}	58^{b}	68^{b}	61^{b}	125^{b}	112^{b}	105^{b}	121^{b}	109^{b}
360	37^{c}	27^{c}	22^{c}	32^{c}	25^{c}	148^{a}	136^{a}	124^{a}	145^{a}	129^{a}
540	28^{d}	22^{d}	17^{d}	27^{d}	19^{d}	49^{d}	33^{d}	25^{d}	42^{b}	30^{d}

PP: Puteh Perak, Mah: Mahsuri, B-370: Bassmati-370, NB: Nona Bokra, and KG: Khari Gnja. Different letters indicate significant difference among accessions using Duncan's multiple range test at $p \leq 0.05$.

Table 6. Mean comparison of the relative growth of cell suspensions in one-step and multiple-step salinity treatment on the basis of dry weight (%).

NaCl	One-step NaCl treatment					Multiple-step NaCl treatment				
(mM)	PP	Mah	B-370	NB	KG	PP	Mah	B-370	NB	KG
0	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{c}	100^{c}	100^{c}	100^{c}	100^{c}
70	62^{b}	47^{b}	42^{b}	59^{b}	45^{b}	123^{b}	115^{b}	107^{b}	119^{b}	112^{b}
140	29^{c}	22^{c}	18^{c}	27^{c}	20^{c}	141^{a}	130^{a}	117^{a}	138^{a}	123^{a}
210	19^{d}	14^{d}	9^{d}	16^{d}	13^{d}	40^{d}	29^{d}	20^{d}	37^{d}	25^{d}

PP: Puteh Perak, Mah: Mahsuri, B-370: Bassmati-370, NB: Nona Bokra, and KG: Khari Gnja. Different letters indicate significant difference among accessions using Duncan's multiple range test at $p \leq 0.05$.

Plant regeneration of the five rice cultivars from callus and cell suspension in three different culture media: The ANOVA results insinuated the significant effects ($p \leq 0.01$) of cultivar and media, as well as their interactions on the plant regeneration of both callus and cell suspensions in this study (Table 7). The mean comparisons showed that there are significant differences among the used media and cultivars in terms of plant regeneration ($p \leq 0.05$), so that the MS media served as the best culture media for plant regeneration of the calli while the LS media produced the lowest callus. Moreover, it was indicated that the

cultivar B-370 and KG produced the highest and lowest rates of plant regeneration on the solid MS media. Interestingly, the results of plant regeneration of the five cultivars in the cell suspension part was almost the same, whereas the liquid MS and B5 media resulted in the highest and lowest regeneration, respectively. Likewise, the cultivars B-370 and KG produced the highest and lowest rates of plant regeneration in the cell suspensions.

Plant regeneration of the five rice cultivars from callus and cell suspension under one-step salinity: According to the ANOVA results, the effects of one-step salinity and cultivars, as well as their interactions were all significant ($p \leq 0.01$) on the plant regeneration of the five cultivars from both callus and cell suspension (Table 8).

Table 7. Analysis of variance of callus and cell suspension regeneration of five rice cultivars using three different media.

S.O.V	df	Mean square	
		Plant regeneration-tissue culture	Plant regeneration-cell suspension
Media (M)	2	3120**	3548.022**
Cultivar (cv.)	4	2570**	1286.022**
M × cv.	8	20**	95.522**
Error	28	0.8	4.894
Total	42	-	-

** The mean difference is significant at the 1 % level.

Table 8. Analysis of variance of plant regeneration of five rice cultivars in solid and liquid MS media under one-step salinity stress condition.

S.O.V	OS plant regeneration-tissue culture		OS plant regeneration-cell suspension	
	df	Mean square	df	Mean square
Salinity (S)	6	14958.006**	5	12946.667**
Cultivar (cv.)	4	1763.72**	4	2641.667**
S × cv.	24	315.387**	20	571.667**
Error	78	1.305	58	0.384
Total	112	-	87	-

OS: One-step salt treatment.

However, the mean comparisons revealed that under the one-step salinity stress condition, the cultivars PP and KG resulted in the highest and lowest plant regeneration from both callus and cell suspension (Figures 7 and 8).

Plant regeneration of the five rice cultivars from callus and cell suspension under multiple-step salinity: Finally, the ANOVA results confirmed that the responses of the five cultivars in terms of plant regeneration from both callus and cell suspension were significantly different ($p \leq 0.01$) to the multiple-step salinity stress (Table 9).

Table 9. Analysis of variance of plant regeneration of five rice cultivars in solid and liquid MS media under multiple-step salinity stress condition.

S.O.V	MT plant regeneration-tissue culture		MT plant regeneration-cell suspension	
	df	Mean square	df	Mean square
Cultivar (cv.)	4	74.1**	4	16509.6**
Error	8	0.2	8	0.4
Total	12	-	12	-

MT: Multiple-step salt treatment.

As a remarkable point and considering the mean comparison results, the superiority of the cultivar PP in terms of plant regeneration from both callus and cell suspension was confirmed under the multiple-step salinity stress condition, as well. Similar to the experiment 6, the cultivar KG produced the lowest rate of plant regeneration from callus and cell suspension under the multiple-step salinity condition (Figure 9 and 10).

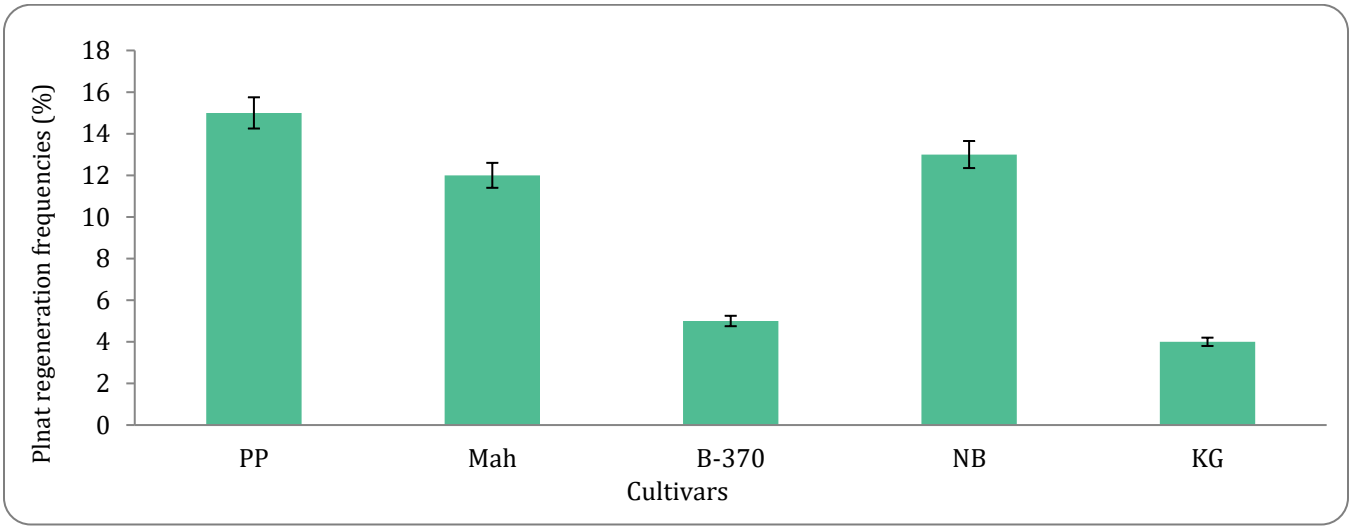

Figure 9. Plant regeneration obtained from multiple-step NaCl treated calli of the five rice cultivars. Puteh Perak (PP), Mahsuri (Mah), Bsmati-370 (B-370), Nona Bokra (NB) and Khari Gunja (KG).

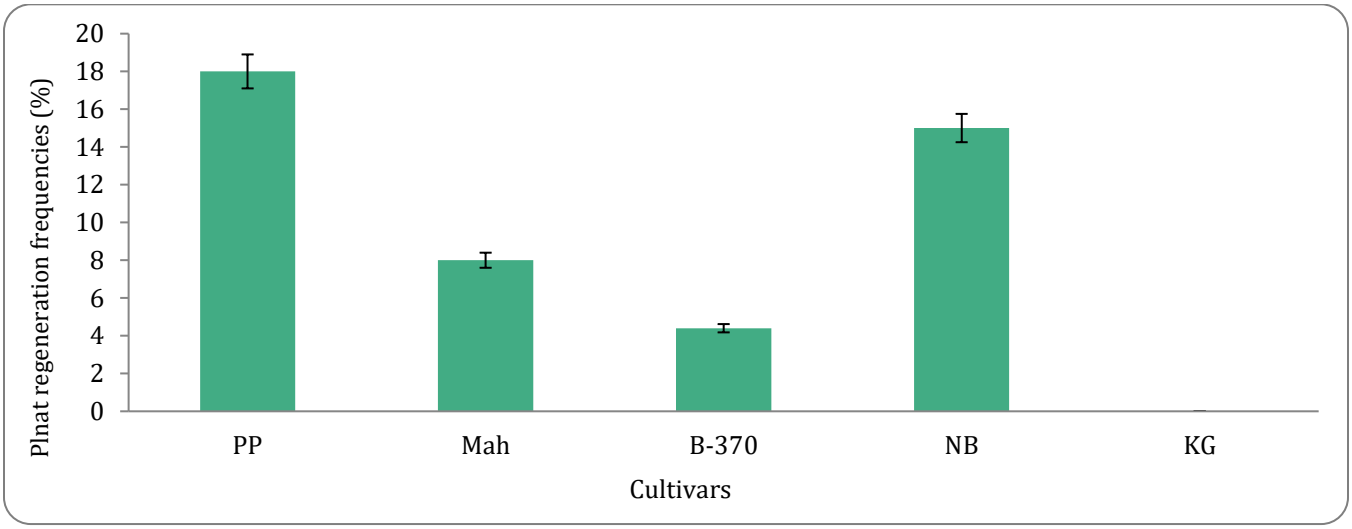

Figure 10. Plant regeneration obtained from multiple-step NaCl treated cell suspensions of the five rice cultivars. Puteh Perak (PP), Mahsuri (Mah), Bsmati-370 (B-370), Nona Bokra (NB) and Khari Gunja (KG).

DISCUSSION

Callus initiation and auxin concentration: Specific auxin to cytokinin ratios in plant tissue culture medium produces an unorganized growing and dividing mass of callus cells in various plant species. In this regard, the results of the present study confirmed that the rate of callus induction in solid MS and liquid B5 media critically depend on the concentration of auxin. Fan *et al*., 2012 states that mechanistically, boosting callus induction could be attributed to upregulation of Lateral Organ Boundaries Domain transcription factors (*LBDs*) by callus-inducing medium (CIM). Similar to our outcomes, it has been reported that most of auxin treatments are capable of increasing callus induction in wheat (*Triticum aestivum* L.) nevertheless, there are rare types of

auxins that have not led to increasing the callus induction. Probably, 2-(2-methyl-4-chlorophenoxy) propionic acid (2-MCPP) is a proper example of this approach (Mendoza and Kaeppler, 2002).

Callus initiation, plant regeneration and culture media optimization: However, media such as the SK-1 medium have specifically been developed for tissue culture of indica rice, more recently (Raina *et al*., 1989 Lentini *et al*., 1995), but the MS (Murashige and Skoog, 1962), B5 (Gamborg *et al*., 1968), LS (Linsmaier and Skoog, 1965), and N6 (Chu *et al*., 1975) culture media have classically been used for callus induction in rice tissue and cell culture. Despite the popularity of the MS media, different researches have resulted in different outcomes depend on the plant species and the types of explants. Ge *et al*., (2006) have introduced the N6 media as an appropriate culture medium for indica rice, while Ullah *et al*., (2007) has indicated that the best calli of indica rice (var. Bas-385) were emerged on both MS and N6 media.

In this study, variations in the callus induction and cell suspension initiation attributed to cultivars has a correlation with plant regeneration.

The capacity for callus formation, cell suspension initiation and plant regeneration was high in B- 370, PP, Mah and NB while it was low in KG. Thus, this study strongly suggests that there might be a close correlation between callus formation, cell suspension initiation and plant regeneration capacity. However, the callus growth of Mah was quite good, but cell suspension growth was quite low. This may be due to the effect of media because we used MS media for callus and B5 for cell suspension, which may be suitable for the other cultivars but not for Mah. The other option is that MS regeneration media may not be suitable for plant regeneration of Mah.

Similarly, Khatun *et al*., (2003) provided strong evidences indicating the MS media as the best callus induction alternative for indica rice. Moreover, there are reports indicating the suitability of the MS media for callus induction in japonica rice (Lee *et al*., 2002) and other cereals such as wheat (Yu *et al*., 2008). Seemingly, the leading role of the MS media in rice tissue culture researches could be attributed to its vitamin, as well as the macro- and micro-elements contents. Despite, different supplementations can effectively affect the performance of these media in the case of callus initiation and cell suspension.

Duration and trend of salinity in tissue culture and plant regeneration: The deleterious effects of salinity on plant growth and development can be justified with various mechanisms like low osmotic potential of soil solution (water stress), nutritive imbalance, specific ion effect (salt stress), or a combination of these factors (Gómez-Cadenas *et al*., 2003). Exactly, the same mechanisms can lead to a malfunction of *in vitro* cell and callus initiations. In addition, the present study can be considered as an *in vitro* selection to save the time required for developing the salt-tolerant rice lines. In another word, since, similar responses to salt stress are expected to be observed in the mature plants, the explants obtained through *in vitro* procedure can be considered as the mini-replicas of a complete field experiment with the ability to stimulate the consequences of salt stress in the whole plant (Cano *et al*., 1998). In the view of the present results, it is concluded that under the same level of salinity, differences in fresh weights of the callus and cell suspensions exist among the five rice cultivars. Hence, the behavior of the rice cultivars against the duration and trend of salinity (one-step and multiple-step) is affected by their genetic ability to tolerate the stress condition.

The investigation of NaCl effect on the callus growth of the five rice cultivars under one-step treatment showed that the dry weight of all cultivars declined with increasing the NaCl in the media. The mentioned decrease was tense in the Mah, B-370 and KG cultivars compared to PP and NB. This study showed that the cell suspension growth was low on the NaCl containing media than NaCl free media. On the other hand, the growth of multiple-steps NaCl treated cell suspension was higher than the one-step NaCl treatment. However, it has been stipulated that salinity in general decreases the rate of callus and cell suspension initiations rates, but the conclusions on the impact of one-step and multiple-step NaCl induction are controversial. In this regard, Blits *et al*., (1993) demonstrated that no callus was initiated when the excised tissue was transferred directly to the medium containing 120 mM NaC1. However, the same results observed when callus grown on normal media (non- saline) and then transferred to the media with 120 mM NaC1. In contrast, Hasson and Poljakoff-Mayber (1995) showed that the callus of *Kosteletzkya virginica* L. (Malvaceae) can growth in the media supplemented with 200 mM NaCl if it is already

adopted stepwise. A justification for the better performance of the calli and cell suspension initiation of the five rice cultivars in multiple-step salt induction is that by the gradual application of NaCl the cells got an opportunity to adapt the stress condition, while such an opportunity was obviously lacking under the one-step NaCl induction. In other words, gradual rise of salinity, especially in higher salinities is a strategy to avoid any osmotic shock on plants (Moseki 2007). Such an event can be attributed to the efficient function of the sodium-potassium pump in expelling the Na^+ ions from the cells in the event of multiple-step salinity (Volkmar *et al*., 1997). Depending on the mode of NaCl application, whether gradual (multiple-step) or in a one-step, plants may experience either salt stress or salt shock, respectively (Shavrukov, 2012). Logically, this fashion can be generalized to callus and cell suspension condition, as well. In addition, patterns of gene expression are different in response to one-step and multiple-step salinity. Therefore, imposition of salt stress by gradual exposure to NaCl (multiple-step) rather than salt shock with a single (one-step) application of NaCl is also suggested for genetic and molecular studies, because this reflects the natural tendency of salinity more closely (Shavrukov, 2012). Salt stress initiates relatively smooth changes in gene expression in response to osmotic stress and a more pronounced change in expression of significant numbers of genes related to the ionic phase of salt stress. The mentioned concept, is being used in advanced technologies pertaining to the comparison between moving bed-membrane bioreactor (MB-MBR) and membrane bioreactor (MBR) systems in which the gradual increase of salinity led to a good acclimation of the biomass as confirmed by the respirometric tests (Di Trapani *et al*., 2014).

In accordance with the present outcomes, another study on some Malaysian rice varieties have revealed a decrease in terms of cell growth and the rate of necrosis in the rice genotypes such as MR219, MR219-4 and MR219-9 under salinity condition. Nevertheless, different feedbacks had been recorded, whereas the variety MR219 responded significantly better than the other varieties. Remarkably, a part of the better *in vitro* callus and cell initiation of the superior rice varieties in response to salinity has been attributed to a higher level of proline accumulation in them (Htwe *et al*., 2011). The role of proline accumulation in mediating the callus response to drought stress appears to be central to the drought tolerance, as well (Al-Taha, 2013). Considering the important role of proline in increasing the level of salt tolerance, the five rice cultivars can be subjected to experiments focused on measuring the accumulation of this amino acid in the studied cultivars.

Mechanistically, to maintain homeostasis during the stress situation, plants have to adjust the internal osmotic conditions and adapt to the changes of osmotic pressure inside the cells. Some systems have proven successful in the study of the salinity problem, but the approach of cell culture technique has been shown to be more effective in selecting salt tolerant lines. At the same time, cell suspension cultures are particularly suitable for physiological, biochemical and molecular cellular response studies. The salt tolerance or sensitivity of the plant is the result of fundamental differences in cellular level adaptations. One approach to studying cellular mechanisms of tolerance is to use undifferentiated cell cultures.

In vitro plant regeneration is always the most critical step for increasing the implementation percentage of various biotechnological techniques and strategies used in plant development programs. In this regard, induction of adventitious roots and shoots, as well as their regeneration from embryogenic callus cultures by using various PGRs are of importance for somaclonal variation (Horn, 1992). *In vitro* plant regeneration in rice has been obtained from different types of explants. However, embryogenic callus and the regenerated plants obtained from mature seed embryo have efficiently been used in *Indica* rice transformation. Despite, noticeable variations are observed in somatic embryogenesis, embryogenic callus production and subsequently in plant regeneration from various origins (Kant *et al*., 2007 Kumar *et al*., 2010).

Since, plant regeneration was performed using a single source viz. embryogenic callus in the present research, making any comparison with respect to the origin of tissue is impossible. Although, the effect of culture media, PGRs and salinity can be subjected to further debates instead. According to the results of the present investigation, the plantlets derived from both callus culture and cell suspensions were highly performant on the solid MS media in terms of the rate of plant regeneration. The availability of the used PGRs in the case of MS media application could be an explanation

for such an observation. The current study proved that the combination of 3 µM IAA and 40 µM Kinetin has the potential of resulting in the highest rate of plant regeneration in rice. In a comparable attempt, Belarmino (2004) experienced a similar point in which the supplementation of MS media with either 0.5 mg L^{-1} NAA or IAA and 0.5 mg L^{-1} BAP led to a medium enhancement in plant regeneration of upland rice. Thadavong *et al*., (2002) has shared the same experience in glutinous rice by highlighting the point that the MS agar medium supplemented with 1 mg L-1 IAA, 4 mg L-1 BA and 800 mg L-1 casein hydrolysate induced the maximum percentage of regeneration (45.00 %). More interestingly, Rafique *et al*., (2011) achieved a very similar result, so that the rice cultivar B-370 (as an *Indica* rice) appeared to be less responsive than the Japonica rice cultivars to regeneration, yet the MS media attested to be the best for callus formation in all rice cultivars. Likewise, the positive effect of the cytokinin-based PGRs such as BAP on plant regeneration was reconfirmed (Rafique *et al*., 2011). Increment of regeneration under cytokinin-based phytohormones was predictable as these classes of PGRs are capable of promoting cell division and expansion in plant roots and shoots. This phenomenon is called cytokinesis (Zhang *et al*., 2005).

CONCLUSION

The plant regeneration of the five rice cultivars under one-step and multiple-step salinity was almost the same as their callogenesis. However, the plant regeneration was reduced with the increase in NaCl concentration, but the intensity of the reduction in the rate of plant regeneration under one-step salinity imposition was more than the multiple-step procedure. Similarly, the growth of callus and cell suspension was also reduced. The mentioned correlation may be due to the deleterious effects of NaCl on cell morphology that may not be clear in growth. Enhancement of the efficient tissue/cell culture protocols for somatic embryogenesis would accelerate the plant breeding programs (Abiri *et al*., 2017). Apparently, the improved somatic embryogenesis protocols and genoproteomics-assisted platforms are the key elements of the next generation plant breeding (Valdiani *et al*., 2017).

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REFERENCES

Abiri R, Maziah M, Shaharuddin NA, Yusof ZNB, Atabaki N, Hanafi MM, Sahebi M, Azizi P, Kalhori N, Valdiani A. 2017. Enhancing somatic embryogenesis of Malaysian rice cultivar MR219 using adjuvant materials in a high-efficiency protocol. *International Journal of Environmental Science and Technology*, 14(78), 1-18.

Al-Taha H. 2013. Effect of shock and gradual drought by PEG on callus growth and proline accumulation in sour orange (*Citrus xaurantium*). *Advances in Agriculture and Botanics*, 5, 77-83.

Belarmino M. 2002. Promotive factors on callus initiation and plant regeneration in upland rice. *Annals of Tropical Resource*, 24, 90-112.

Binh D Q, Heszky L E, Gyulai G, Csillg A. 1992. Plant regeneration of NaCl pretreated cells from long term suspension culture of rice (*Oryza Sativa L.*) in high saline condition. *Plant Cell Tissue Organ Culture*, 29, 75-82.

Blits K C, Cook D A, Gallagher J L. 1993. Salt tolerance in cell suspension cultures of the halophyte Kosteletzkya virginica. *Journal of Exprimental Botany*, 44, 681-6.

Cano E A, Perez-Alfocea F, Moreno V, Caro M, Bolarin M C. 1998. Evaluation of salt tolerance in cultivated and wild tomato species through *in vitro* shoot apex culture. *Plant Cell Tissiue Organ Culture*, 53, 19-26.

Chinnusamy V, Jagendorf A, Zhu J K. 2005. Understanding and improving salt tolerance in plants. *Crop Science* 45, 437-48.

Chu C C, Wang C S, Sun C S, Hsu V, Yin K C, Chu C Y, Bi F Y. 1975. Establishment of an efficient medium for anther culture of rice through experiments on the nitrogen source. *Scientia Sinica*, 18, 659-68.

Croughan T P, Stavarek S J, Rains D W. 1981. *In vitro* development of salt resistant plants. *Environmental and Experimental Botany*, 24, 317-24.

Delporte F, Mostade O, Jacquemin J M. 2001. Plant regeneration through callus initiation from thin mature embryo fragments of wheat. *Plant Cell Tissiue Organ Culture*, 67, 73-80.

Di Trapani D, Di Bella G, Mannina G, Torregross M, Viviani G. 2014. Comparison between moving bed-membrane bioreactor (MB-MBR) and membrane bioreactor (MBR) systems: influence of wastewater salinity variation. *Bioresource Technology*, 162, 60-9.

Fan M, Xu C, Xu K, Hu Y. 2012. Lateral organ boundaries domain transcription factors direct callus formation in *Arabidopsis* regeneration. *Cell Research*, 22, 1169-80.

Gamborg O L, Miller R A, Ojima K. 1968. Nutrient requirements of suspension culture of soybean roots cells. *Experimental Cell Research*, 50,150-8.

Ge X, Chu Z, Lin Y, Wang S. 2006. A tissue culture system for different germplasms of *indica* rice. *Plant Cell Reportes*, 25, 392-402.

Gómez-Cadenas A, Arbona V, Jacas J, Primo-Millo E, Talon M. 2003. Abscisic acid reduces leaf abscission and increases salt tolerance in citrus plants. *Journal of Plant Growth Regulation*, 21, 234-40.

Hasson E, Poljakoff-Mayber A. 1995. Callus culture from hypocotyls of *Kosteletzkya virginica* (L.) seedlings. *Plant Cell Tissue Organ Culture*, 43, 279-85.

Heszky L E, Nam S, Horvath Z S. 1986. Rice tissue culture and application to breeding. 2. Factors effecting the plant regeneration during subculture of diploid and haploid callus. *Cereal Research Communication*, 14, 289-96.

Hiei Y, Komari T. 2006. Improved protocols for transformation of *indica* rice mediated by *Agrobacterium tumefaciens*. *Plant Cell Tissue Organ Culture*, 85, 271-83.

Htwe N N, Maziah M, Ling H C, Qamaruz-Zaman F, Zain A M. 2011. Responses of some selected Malaysian rice genotypes to callus induction under *in vitro* salt stress. *African Journal Biotechnology*, 10, 350-62.

Kant P, Kant S, Jain R K, Chaudhury V K. 2007. Agrobacterium mediated high frequency transformation in dwarf recalcitrant rice cultivars. *Biologia Plantarum*, 5, 61-8.

Kavi Kishor P B, Reddy G M. 1986. Regeneration of plants from long term cultures of *Oryza sativa* L. *Plant Cell Reports*, 5, 391-3.

Kavi Kishor W X, Mehta A R. 1989. Carbohydrate oxidation during organogenesis in callus cultures of tobacco. *Indian Journal of Expimental Biology*, 27, 124-7.

Khatun M M, Ali M H, Desamero N V. 2003. Effect of genotype and culture media on callus formation and plant regeneration from mature seed scutella culture in rice. *Plant Tissue Culture*, 13, 99-107.

Kumar V, Shriram V, Kavi Kishor P B, Jawali N, Shitole M G. 2010. Enhanced proline accumulation and salt stress tolerance of transgenic *Indica* rice by over-expressing P5CSF129A gene. *Plant Biotechnology Reports*, 4, 37-48.

Kumar V, Shriram V, Nikam T D, Kavi Kishor P B, Jawali N, Shitole M G. 2008. Assessment of tissue culture and antibiotic selection parameters useful for transformation of an important *Indica* rice genotype Karjat-3. Asian Australasian *Journal of Plant Science and Biotechnology*, 2, 84-7.

Lee K, Jeon H, Kim M. 2002. Optimization of a mature embryo-based *in vitro* culture system for high-frequency somatic embryogenic callus induction and plant regeneration from japonica rice cultivars. *Plant Cell Tissue Organ Culture*, 71, 237-44.

Zhang Q. 2005. Optimising the tissue culture conditions for high efficiency transformation of *indica* rice. *Plant Cell Reports*, 23, 540-7.

Linsmaier E M, Skoog F. 1965. Organic growth factor requirements of tobacco tissue cultures. *Physiologia Plantarum*, 18, 100-27.

Mendoza M G, Kaeppler H F. 2002. Auxin and sugar effects on callus induction and plant regeneration frequencies from mature embryos of wheat (*Triticum aestivum* L.) *In Vitro Cellular and Devlopmental Biology-Plant*, 38, 39-45.

Moseki B. 2007. Evidence for the presence of two components of the root transmembrane potential of a halophyte *Sesuvium portulacastrum* L. grown under saline conditions. *Scientific Research Essays*, 2,13-15.

Munns R, Tester M. 2008. Mechanisms of salinity tolerance. *Annul Review of Plant Biology*, 59, 651-81.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Plant Physiology*, 15, 473-97.

Rafique M Z, Rashid H, Chaudhary M F, Chaudhry Z, Cheema N M. 2011. Study on callogenesis and organogenesis in local cultivars of rice (*Oryza sativa* L.). *Pakistan Journal of Botany*, 43, 191-203.

Raina S K, Balachandran S M, Virmani S S, Zapata F J. 1989. An improved medium for efficient anther culture of some *indica* rice hybrids. *International Rice Research News*, 14, 3-4.

Sahoo K K, Tripathi A K, Pareek A, Sopory S K, Singla-Pareek S L. 2011. An improved protocol for

efficient transformation and regeneration of diverse *indica* rice cultivars. *Plant Methods*, 7, 49-59.

Shavrukov Y. 2013. Salt stress or salt shock: which genes are we studying? *Journal Expimental Botany*, 64, 119-27.

Suenaga K, Abrigo E M, Yoshida S. 1982. Seed-derived callus culture for selecting salt-tolerant rice. IRRI research paper series, No. 79. International Rice Research Institute, Manila, Philippines.

Thadavong, S. Sripichitt, P. Wongyai, P. Jompuk, P. 2002. Callus induction and plant regeneration from mature embryos of glutinous rice (*Oryza sativa* L.) cultivar TDK1. *Kasetsart Journal Natural Science*, 36, 334-44.

Ullah H, Ullah I, Jadoon S A, Rashid H. 2007. Tissue culture techniques for callus induction in rice. *Sarhad Journal of Agriculture*, 23, 81-6.

Vasil I K, Thorpe T A. 1994. Plant cell and tissue culture. Dordrecht: Kluwer Academic Publishers.

Volkmar K M, Hu Y, Steppuhn H. 1997. Physiological responses of plants to salinity: A review. *Canadian Journal of Plant Science*, 78, 19-27.

Wanichananan P, Teerakathiti T, Roytrakul S, Kirdmanee C, Peyachoknagul S. 2010. A highly efficient method for *Agrobacterium* mediated transformation in elite rice varieties (*Oryza sativa* L. ssp. *indica*). *African Journal of Biotechnology*, 9, 5488-95.

Yu Y, Wang J, Zhu M L, Wei Z M. 2008. Optimization of mature embryo-based high frequency callus induction and plant regeneration from elite wheat cultivars grown in China. *Plant Breeding*, 127, 249-55.

Zeng L, Shannon M C, Griee C M. 2002. Evaluation of salt tolerance in rice genotypes by multiple agronomices parameters. *Euphytica*, 127, 235-45.

Valdiani A, Talei D, Lattoo SK, Ortiz R, Rasmussen SK, Batley J, Rafii MY, Maziah M, Sabu KK, Abiri R, Sakuanrungsirikul S, Tan SG. 2017. Genoproteomics-assisted improvement of *Andrographis paniculata*: toward a promising molecular and conventional breeding platform for autogamous plants affecting pharmaceutical industry. *Critical Reviews in Biotechnology,* 37 (6), 803–816.